ROSCOE B. JACKSON MEMORIAL LABORATORY

Bar Harbor, Maine

September 18, 1958
#4

Progress Report

to

Tobacco Industry Research Committee
150 East Forty-second St.
New York 17, N.Y.

Title of Project:

The production of genetically controlled animals and tumors for possible use by T.I.R.C. grantees in experimental research on tobacco in relation to health by (a) expansion of known inbred stocks and sources of tumor supply; (b) the production of such hybrids or heterozygous types as become necessary; and (c) the relation of this material to specific experimental work at the Laboratory.

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Period: November 1, 1957 - September 30, 1958

As demonstrated in the first three progress reports of work done under this grant, the breeding colonies of the listed inbred stocks of mice and their hybrids have been undergoing continuous expansion.

The rate of this increase is summarized and extended through the fourth year as Exhibit A. While this expansion has been carried on as rapidly as the breeding colonies and available housing facilities would permit, the production from the colonies has never caught up with the demand for these mice. Hence, the expansion continues through the fifth year as illustrated by Exhibit B. It is expected that this enlargement of the colony will be accomplished in the late spring of 1959.

These expansions, with the concentrations of populations which they have entailed, have created many problems in:

a) Maintenance of genetic purity of strain,
b) Health control,
c) Housing arrangements and housekeeping, and
d) Personnel training.

Genetic purity of strain has been safeguarded in the following manner:

a. Maintenance of a segregated colony of foundation stocks,
b. A carefully controlled and studied colony in which an expansion in numbers is carried on,
c. Systematic recording of the breeders obtained by the production colony from the expansion colony, and
d. Keeping animals used in final production pens as close as possible, in generations, to the original pair in the foundation stock.

Health control has been accomplished by:

a. Systematic observation and testing of colonies for diseases indigenous to mice,
b. Systematic treatment for mite control,
c. Physical segregation of breeding colonies,
d. Rigid culling of weaklings,
e. Conversion from wooden to stainless steel breeding pens, and
f. Improved washing and sterilization techniques.

Housing and housekeeping:

The effort to find suitable space and to adapt it to our use has been continuous. This has resulted in the acquisition of five outlying buildings; arranging for heating, ventilating and waste disposal, and in the training of personnel in the necessary techniques and indoctrination in the necessary safeguards to the health of the animals.

As a result of these efforts and precautions we have been able to

keep the colonies in good health, provide breeders for renewing the old pens and for the scheduled expansion, conduct a rigid culling program, and provide others with 78% of the total production.

We are happy to report that this program is now self-supporting and that a request for further support from T.I.R.C. is no longer justified.

In addition to the above, the funds in the grant have been used to support the work of Dr. R. H. Gwynn in the "Biological activity of tobacco tars."

Dr. Gwynn's report:

During the past year the following investigations were made regarding the biological activity of Tobacco Tars*.

Effects of Tobacco tars on mouse lung and subcutaneous tissues. While there is considerable information available concerning the effects of tobacco tar on mouse skin, very little is known about its action on other tissues. For this reason the following experiments were undertaken.

a) Denicotinized tobacco tar was prepared by the method of Wynder et al. (1) and dissolved in carbowax. Small amounts of this mixture were injected into the thoracic cavities of one group of mice and into the bronchi of another. (The tar was introduced into the bronchi by means of a needle passed down the trachea.) In both cases the mice died soon after treatment. Mice treated with the solvent alone survived.

b) Lungs from 17-19 day old A/sn embryos were grafted into the axillary region of young adult A/sn mice. Two months later 40 of these grafts were threaded with silk thread which had been soaked in denicotinized tobacco tar, and 20 were threaded with untreated thread. Hosts carrying grafts with tarred threads showed symptoms of severe nicotine poisoning but recovered in a few hours. 6 months after threading hosts bearing 20 tar treated grafts were killed. No tumours were found. The remaining grafts will be examined when they have been exposed to the action of tarred threads for 12 months.

c) 0.1 ml of a mixture consisting of 2 parts of denicotinized tar and 1 part of sesame oil was injected subcutaneously into 20 A/sn mice. These were examined once each month, but no tumours have yet been found. This experiment is still in progress.

* The Tobacco tars used in the experiments described in this report should more properly be called cigarette smoke condensates, but the shorter term is more convenient.

Effects of tobacco tars on the sterols of mouse skin. The sterols present in mouse skin may be roughly divided into two categories, Cholesterol and the "fast acting" sterols. Though small amounts of 7-dehydrocholesterol and other sterols of unknown constitution may be present, the major constituent of the "fast acting" sterol fraction is Δ^7-cholestenol (2-4), therefore, in this report the whole "fast acting" fraction will be referred to as Δ^7-cholestenol.

It has been shown by Baumann, Kandutsch and their co-workers (2-4) that the application of carcinogenic hydrocarbons to the skin of mice causes a drop in the concentration of Δ^7-cholestenol, whereas only a very few non-carcinogenic irritants have this effect. Since some workers have shown that tobacco tars are carcinogenic to mouse skin, and it is suspected that active fractions are polycyclic hydrocarbons, the effect of tobacco tar on the sterols in the skin was studied. The ultimate object of this study was to see if changes in sterol level could be used as a test for carcinogenic activity in fractions of tobacco tars.

The tar used in these experiments was made by smoking cigarettes in a simple machine. Since these experiments were of an exploratory nature no attempt was made to measure the combustion temperature of the cigarettes. The rate of smoking was two-second puffs per minute, and the yield of tar was about 4 grams per hundred cigarettes. For application to mice the tar was made up as a 50% solution in acetone (w/v). The mice used throughout these experiments were BAF_1 males, 8-10 weeks old. Tar was applied to the back, after the hair had been removed with electric clippers, using a brush which deposited 40 mg. of tar per application. Controls received acetone only. Mice were killed in various intervals after tar applications and a measured area of skin removed for sterol estimation. Total sterols were measured by the method used by Baumann (2). Sterols of experimental and control mice were always estimated at the same time. Six mice were used for each estimation. Pieces of skin adjacent of the measured area were taken for microscopical examination.

Although the concentration of Δ^7-cholestenol was always depressed by the application of tobacco tar, the degree of depression varied from experiment to experiment even when the same batch of tobacco tar was used. This appeared to be due to differences between different batches of mice, differences that require further investigation. In spite of these variations certain consistent effects were found. These are described below.

a) A single application of 40 mgs. of tar caused a depression in the concentration of Δ^7-cholestenol, the maximum depression appeared between 3 and 5 days after the application (Table 1, group a). The concentration had returned to normal by the 9th day.

b) Two applications, each of 40 mgs., given on the same day, were no more effective than a single application. (Table 1,b)

c) Two applications, each of 40 mgs, given on consecutive days produced a greater depression in Δ^7-cholestenol concentration than did a single application and the concentration was still depressed on the 9th day. (Table 1. c)

d) Multiple applications of tobacco tar appeared to cause less depression in the concentration of Δ^7-cholestenol than did a single or two applications. When 4 applications were made over a period of 10 days and sterol content estimated on the 11th day a 24% depression in Δ^7-cholestenol concentration was found. However, when 8 applications were made over a period of 17 days no depression in the concentration of this sterol was observed. A somewhat similar observation has been made when carcinogenic hydrocarbons are applied repeatedly, but here the depression in Δ^7-cholestenol concentration persists for a greater length of time.

e) The degree of depression produced by two tobacco tars produced under different conditions was about the same. Tar (H) was produced by smoking cigarettes as rapidly as possible in the machine, while tar (S) was produced by smoking cigarettes as slowly as possible, with a degree of suction just sufficient to keep the smoke flowing into the machine. This would appear to indicate materials responsible for causing depressions in the concentration of Δ^7-cholestenol of the skin are produced even at the lowest rate of cigarette combustion. (Table 1, eH, eS).

f) The effect of Iodacetic acid, (a strong irritant for mouse skin) and β-propiolactone (a weak carcinogen) on the sterol concentration were examined. Neither caused a significant depression in the Δ^7-cholestenol concentration, although the former causes histological changes very similar to those produced by tobacco tars. (Table 1, fI, fP)

As previously stated, the degree of change in the sterol concentration after tar applications to the skin is somewhat variable. It was suspected that this might be in some way related to the state of the skin at the time of tar application. For this reason the sterol concentration of the skin was estimated at various stages in the hair cycle.

When the hair is plucked from the backs of mice during the resting phase of the hair cycle, a new cycle begins at once. Estimations of the sterol content of mouse skin taken at intervals after plucking showed that both cholesterol and Δ^7-cholestenol concentrations rose rapidly reaching a maximum at 7 days after plucking (Table 2) and thereafter declined to the normal value. Further investigation of this effect is required to discover its significance in connection with effects produced by tobacco tar.

While these experiments have yielded some interesting information

and suggest that changes in the concentration of 7-cholestenol may be used as a means of detecting carcinogenic activity in materials applied to the skin, considerable effort is required to elucidate the significance of this sterol in carcinogenesis.

Studies on enzyme activities in mice receiving carcinogenic and other treatments. The effect of carcinogens on enzyme systems in vivo has received little study. Apart from the importance of these systems in tumor biology, it is clear that if one or more enzymes could be shown to be specifically affected by the action of carcinogens an extremely useful means of detecting carcinogenic activity in suspect materials would be at hand. With the aim of developing such a test the effect of carcinogens on a number of enzymes in mice was investigated.

It has been shown by Adams and Roe (5) that the activity of liver catalase is depressed in mice treated with some carcinogens. Since the activity of this enzyme is also depressed by the presence of a growing tumor in the mouse, it seemed possible that other enzymes which are affected by the presence of a transplanted tumor might also be affected by carcinogens. Livers of mice bearing transplanted tumors show decreased arginase and esterase activity. Therefore, the effect of carcinogens on these enzymes as well as catalase was studied.

The carcinogens used were 9.10-dimethyl-1.2-benzanthracene, 20-methylcholanthrene and croton oil. (The last is variously described as a promoting agent or a very weak carcinogen.) A single application of each was made to the mice used. Estimations of enzyme activity in the livers were made at 2, 3 and 4 days after treatment. From these studies the following points emerged:

a) It was confirmed that mice bearing transplanted tumors showed depressed levels of all three enzyme activities.

b) Neither 9.10-dimethyl-1.2-benzanthracene nor 20-methylcholanthrene caused any depression in activity of these enzymes in the livers of the mice used in these studies. (A/sn, C57BL and BAF_1 males.)

c) Croton oil depressed the liver catalase activity of C57BL mice, and to a less extent in BAF_1 and A/sn mice. The other enzymes were not affected. (Table 3)

These results suggest that there is no direct connection between carcinogenic activity and catalase depressing action, nor is there any obvious connection between effects produced by transplanted tumors and those produced by carcinogenic materials, on the enzymes of the liver.

Experiments in progress and proposed studies.

I) Studies on the effect of Tobacco tars on skin sterols.
II) Studies on the effect of carcinogens on enzymes of the skin and lungs.

III) Comparison of other biochemical changes produced in mouse tissues by carcinogenic hydrocarbons and those produced by tobacco tars.

IV) Further attempts to induce tumours of the lungs and subcutaneous tissues with tobacco tars.

V) Examination of the susceptibility of sub-lines of A strain mice to the induction of lung tumours by carcinogens. (Preliminary experiments suggest that the sub-lines A/sn, A.sw, A.by and their hybrids differ considerably in their sensitivity to the lung tumour inducing action of 1.2.5.6.-dibenzanthracene).

References:

1) Wynder, E. L. and Wright, G. A study of tobacco carcinogenesis, I. The primary fractions. Cancer 10:255, 1957.

2) Miller, W. L. and Baumann, C. A. Skin sterols. IV. Distribution of Δ^7-cholestenol. Proc. Soc. Exper. Biol. & Med. 85:561, 1954.

3) Kandutsch, A. A. and Baumann, C. A. Skin sterols. VIII. Effects of carcinogens, co-carcinogens, and of certain hyperplastic agents. Cancer Research 15:128, 1955.

4) Kandutsch, A. A., Murphy, E.D., and Dreisbach, M. E. Effects of hormonal factors and of the hereditary character "hairless" on the sterols of mouse skin. Cancer Research, 16:63, 1956.

5) Adams, D. H. and Roe, F. J. C. The action of some chemical substances on mouse liver catalase activity in vivo. Brit. J. Cancer, 7:509, 1953.

TABLE 1

Effect of Tobacco tar, Iodoacetic acid and β-Propiolactone on the Δ^7-cholestenol concentration of mouse skin.

Group	Material applied	No. of applications	Total amount (Mgs.)	Concentration of Δ^7-cholestenol expressed as a % of control value this number of days after 1st application of material tested. 3	4	5	9	11
a	Tobacco tar	1	40	63	61	67	114	-
b	" "	2 on same day	80	-	52	-	-	-
c	" "	2	80	-	30	-	63	-
d	" "	4	160	-	-	-	-	76
eH	Tobacco tar H	1	40		35	-	-	-
eS	Tobacco tar S	1	40		38	-	-	-
fI	Iodoacetic acid	1	2		101	-	-	-
fP	β-propiol-actone	1	5		78	-	-	-

TABLE 2

Concentration of cholesterol and Δ^7-cholestenol at various stages in the hair cycle of mouse skin.

Days after start of cycle	Δ^7/cm^2 µg.	Δ^7% of control	Δ^5/cm^2 µg.	Δ^5% of control
3	24.1	89	104.8	150
7	70.3	260	205.0	293
12	42.8	158	145.5	208
23	27.1	100	79.9	100

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Δ^7-/cm^2 = micrograms of Δ^7-cholestenol per square cm. of skin

Δ^7% of control = concentration of Δ^7-cholestenol expressed as percentage of control value

Δ^5/cm^2 = micrograms of cholesterol per sq. cm of skin

Δ^5% of control = concentration of cholesterol expressed as a percentage of the control value

TABLE 3

Effect of croton oil on the activity of liver catalase in mice.

Material applied	Amount applied (Mgs.)	Strain of mice	Activity of liver catalase expressed as a percentage of the control value this number of days after treatment. 2	3	4
croton oil	0.4	A/sn	93	65	115
" "	"	C57BL	43	41	50
" "	"	BAF_1	56	-	98
9.10-dimethyl-1.2-benzanthracene or 20-methyl-cholanthrene	0.2	A/sn, C57BL, BAF_1	No significant effect.		

Exhibit A

COMPARATIVE SUMMARY MOUSE PRODUCTION

Strain	1954-55 Produced	1955-56 Produced	1956-57 Produced	1957-58 Produced
C3H	41,557	47,737	48,565	44,627
C57BL/6	6,943	40,409	107,778	209,631
DBA/1	28,006	23,471	16,585	19,335
DBA/2	57,323	63,582	88,593	139,005
BALB/c	18,202	20,972	20,449	16,255
C57BR/cd	4,808	8,590	4,562	3,624
A/Jax	17,423	18,060	21,825	20,789
A/Heston	4,140	7,496	5,610	7,260
AKR	14,659	20,743	25,829	32,653
C57 Leaden	7,432	8,559	6,327	9,237
BAF_1	3,800	11,696	13,124	17,576
CAF_1	21,342	22,391	19,463	20,903
LAF_1	21,960	29,680	28,562	26,974
BBF_1	5,008	15,463	7,409	------
C3HeB	858	3,548	2,937	3,496
$AKD2F_1$	11,075	19,137	12,918	22,793
Swiss	14,409	3,987		
ABC	454			
SWR		1,565	3,348	3,816
CDF_1	514			
RFM		3,433	5,536	3,854
CBF_1		9,940	8,027	------
BDF_1		24,403	145,187	278,897
C58		796	1,470	2,638
$D_2C_3F_1$				5,005
CBA				711
Totals	279,913	405,658	594,104	889,079

Exhibit B

FURTHER EXPANSION OF BREEDING COLONIES

Strain	2/28/58±	Expand to Boxes	Status Sept. 1, 1958	Each Box* Produces per year	Potential Production of Proposed Expansion
C3H	1,098	1,524	1,470	42.1	64,260
C57BL/6	3,394	6,173	4,831	57.2	353,096
DBA_1	378	378	373	56.9	21,508
DBA_2	2,472	4,212	3,805	51.0	214,812
BALB/c	280	560	336	57.0	31,920
C57Br/cd	72	84	84	58.5	4,914
A/Jax	553	553	551	37.6	20,793
A/He	224	224	224	37.0	8,288
AKR	669	787	779	54.0	42,498
C57L	218	218	224	45.3	9,875
BAF_1	168	168	168	104.7	17,590
CAF_1	245	371	315	85.3	31,646
LAF_1	315	350	259	81.7	28,595
C_3HeB	91	175	139	38.4	6,720
AKD_2F_1	217	301	217	105.0	31,605
BdF_1	3,192	3,500	3,412	101.1	353,850
SWR	70	84	70	54.5	4,578
C58	84	224	84	33.8	7,571
RFM	84	84	84	45.9	3,854
$D_2C_3F_1$	154	368	252	29.8	10,966
CBA	19	72	72	21.5	1,548
	13,997	20,410	17,749		1,270,487

* Based on records of 1957-1958

± Each box contains 2 breeding pens